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| Original Article |

G_h Mediates α1-Adrenoceptor-Stimulated Hypertrophy Following Ischemia/Reperfusion in Cardiomyocytes

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Abstract : Ischemia/reperfusion (I/R) has been associated with ventricular (LV) remodeling, including induction of hypertrophy. Stimulation of α_1-Adrenoceptors (ARs) has been implicated in the pathogenesis of cardiac hypertrophy. The present study is to test the hypothesis that α_1-AR-mediated hypertrophy is selectively mediated via the oxidative modification of G_h during hypoxia/reoxygenation (H/R) or I/R. Reactive oxygen species (ROS) was increased in H/R and expression level of membrane G_h. To further address involvement of G_h in hypertrophic response, specific relation of G_h was confirmed by using G_h overexpression and blocking into cardiomyocytes. Mainly increased membrane G_h protein by ROS has a more sensitive effect on myocardial hypertrophy through MEK1,2/ERKs signal transduction pathway, but induction of proto-oncogene except *c-fos* and expression of PLC δ_1 was independent in ROS condition. These results provide that ROS production by I/R mediates G_h protein increment and G_h protein leads to more specific responsiveness to NE-stimulated hypertrophic cardiomyocytes.

Key words: *G_h, α_1-adrenoceptor, ischemia/reperfusion, reactive oxygen species, hypertrophy*

1. Introduction

Ischemic condition is lead to reduce morbidity and mortality when coronary blood supply to myocardium is decreased. Oxygen supply is insufficient to support oxidative phosphorylation of mitochondria, and may be followed by reperfusion.[1] Cardiac hypertrophy is a compensatory process which occurs in pathological conditions and an adaptation process against hemodynamic overload.[2] Hypertrophy should be result in wall thickness, reduction of ventricular lumen, increment of cardiomyocyte length and ventricular luminal size in the heart. The relationship between cardiac hypertrophy and adrenergic activity been documented several reports.[3,4] Adrenoceptors (ARs), members of the G-protein coupled receptor (GPCR), have an important role in interface between sympathetic nerve and the cardiovascular system in the rapid regulation of myocardial function.[5-7] Among α-ARs, the α_1-ARs exist in cardiomyocytes and have been implicated in the pathogenesis of cardiac hypertrophy in ischemia-induced cardiac arrythmias and ischemic preconditioning.[8] Signaling pathways leading from α_1-ARs activation to hypertrophy are complex and may involve activation of extracellular signal-regulated protein kinase (ERK) and PI3K through G_q, PKC, Ras, and activation of calcineurin through Ca^{2+} and calmodulin.[9,10]

G_h (tissue type transglutaminase2) is ubiquitously expressed in mammalian tissues and acts as enzymatically-active transglutaminases and GTP-binding protein mediating intracellular signaling via the α_{1B}-AR.[11] Previous studies indicate that G_h play a main regulator in cell growth and differentiation, apoptosis and tissue repair as well as cardiac hypertrophy by α_1-AR stimulation,[12,13] and participate in the AR signaling pathway to ischemia/reperfusion (I/R) by using knockout mice.[14] Reactive oxygen species (ROS) such as superoxide generated endogenously or in response to environmental stress has been implicated in tissue injury including I/R. Several researchers have shown that ROS could

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link to the hypertrophy in neonatal rat ventricular myocytes as well as in adult rat ventricular myocytes[15,16] and may mediate α-AR-stimulated hypertrophic signaling in adult myocytes via G protein modification.[17] Yet, hypertrophic molecular mechanism of G_h activation induced by ROS in exact I/R heart remains poorly understood.

In the present study, it will be addressed whether production of ROS through I/R or hypoxia/reoxygenation (H/R) injury affect oxidative modification of G_h and lead to NE-stimulated hypertrophy. Here, our data showed that α_1-AR-mediated hypertrophic responses was related to G_h and overexpressed or downregulated G_h regulated to ERK1,2 signal. In addition, I/R or H/R affected to MEK1,2/ERK1,2 signal transduction pathway, while PLC δ_1 and proto-oncogene was independent on NE-stimulated cardiomyocytes.

2. Materials and Methods

2.1 Myocardial Ischemia-Reperfusion Injury

All experimental procedures for animal studies were approved by the Committee for the Care and Use of Laboratory Animals, Yonsei University College of Medicine and were performed in accordance with the Committee's Guidelines and Regulations for Animal Care. Myocardial infarction was produced in male Sprague-Dawley rats (200±50 g) by surgical occlusion of left anterior descending coronary artery, according to the method described previously with minor modifications.[18] Briefly, rats were anesthetized by intramuscular injection of zoletil (50 mg/kg) and xylazine (5 mg/kg), and the chest was opened by cutting the third and fourth ribs. The heart was exteriorized through the intercostal space and the left coronary artery was ligated 2-3 mm from its origin with a 6-0 prolene suture. Coronary occlusion was maintained for 1 h, followed by removal of the suture and reperfusion for 3 h. Throughout the operation, the animals were ventilated with 95% O_2 and 5% CO_2, using a Harvard ventilator (Harvard Apparatus, USA). Sham-operated animals were treated similarly, except that the coronary suture was not tied.

2.2 Culture of Cardiomyocytes and *In Vitro* Hypoxia-Reoxygenation Treatment

Neonatal rat cardiomyocytes were prepared by an enzymatic method. Briefly, hearts of 1- to 2-day-old Sprague-Dawley rat pups were dissected, minced, enzymatically dispersed with 10 ml of collagenase I (0.8 mg/ml, 262 U/mg, Gibco BRL, UK) and centrifuged differentially to yield 5×10^5 cells/ml. After incubation for 4-6 h, the cells were rinsed twice with cell culture medium and 0.1 mM BrdU (Sigma Chemical, USA) was added. Cells were then cultured in a CO_2 incubator at 37°C for 1-2 days. For the treatment of H/R, the cells were incubated for 1 h using deoxygenated serum free α-MEM in an anaerobic chamber (Thermo Forma, USA).

2.3 Measurement of Intracellular Reactive Oxygen Species Generation

Cardiomyocytes were labeled with 10 μM 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Invitrogen Co., USA). The dye, when exposed to an excitation wavelength of 480 nm, emits light at 535 nm only when it has been oxidized. Labeled cells were examined using a luminescence spectrophotometer for oxidized dye.

2.4 Flow Cytometry

Cardiomyocytes were retrieved with a standard trypsinization technique. Cells were washed in phosphate buffered saline (PBS) and fixed in 70% ethanol at 4°C for 30 min with agitation. Cells were washed twice in PBS and resuspended at 2×10^6 cells/ml in blocking buffer (1% BSA, 0.1% FBS) containing anti-G_h antibody (Santa Cruz Biotechnology, USA) diluted at 1/200. The labeling reaction mixture was agitated for 20 min at room temperature. Cells were washed twice and then labeled with anti-goat-FITC conjugated IgG (Jackson ImmunoResearch Laboratories, USA) diluted to 1/400 for 20 min at room temperature in the dark. After two more washes, flow cytometric analysis was performed on FACS Calibur system (Becton Dickinson, USA) using CellQuest™ software with 10,000 events recorded for each sample. Data was acquired in single parameter histogram with appropriate particlesize and light scatter gating.

2.5 Transfection

Transfections of G_h cloned into pcDNA3.1 (Invitrogen) were performed using LIPOFECTAMINE PLUS™ reagent (Invitrogen). Briefly, cardiomyocytes cultured in a 60 mm culture plate (5×10^5 cells/plate) were washed twice with serum-free MEM. LIPOFECTAMINE PLUS™ reagent was diluted with serum-free MEM and combined with 2 μg of DNA for each plate. The DNA and LIPOFECTAMINE PLUS™ complexes were added onto each plate. After 12 h incubation in a CO_2 incubator at 37°C, the medium was changed to 10% FBS-MEM.

2.6 RNA Interference

For function-blocking experiments, we used small interfering RNA molecules (siRNA) targeted at G_h mRNA. A 21-nt sequence for siRNA was derived from the rat G_h

(GenBank accession no. GI: 9507184) and obtained from Ambion (USA): siRNA sequence of G_h (sense: 5'-GGGUUACCGGAAUAUCAUCTT-3' antisense: 5'-GAUGAUAUUCCGGUAACCCTT-3'). Cardiomyocytes were transfected with siRNA by using siPORT NeoFX (Ambion). Briefly, RNA duplex (10 nM of final concentration) was incubated in serum-free α-MEM containing 15 μl of siPORT NeoFX for 10 min. The complex was added to the empty 60 mm culture plate, then overlay cardiomyocytes suspension (1×10^5 cells/plate) onto the culture plate wells containing transfection complexes. The transfected cells were incubated in normal cell culture condition until ready for assay.

2.7 Immunoblot Analysis

Proteins were separated by SDS-PAGE using 10-12% polyacrylamide gels and then electrotransferred to methanol-treated polyvinylidene difluoride (PVDF) membranes. The blotted membranes were washed twice with water and blocked by incubation with 5% nonfat dried milk in PBS buffer (8.0 g NaCl, 0.2 g KCl, 1.5 g NaH_2PO_4, 0.2 g K_2HPO_4 per liter). The membranes were probed with G_h, G_q, G_s, ERK1/2, MEK, PLC-δ1, β-actin, anti-phospho-p38 kinase, -JNK, -ERK1/2 and -MEK antibodies (Santa Cruz Biotechnology), followed by goat anti-rabbit, goat anti-mouse and mouse anti-goat IgG-peroxidase. The blots were detected using enhanced chemiluminescence kits (ECL; GE healthcare, USA).

2.8 RT-PCR Analysis

The mRNA level of proto-oncogene was analyzed by the reverse transcription polymerase chain reaction (RT-PCR) technique. Total RNA was prepared by Ultraspect™-II RNA system (Biotecx Laboratories, USA), and single-stranded cDNA was then synthesized from isolated total RNA by Avian myeloblastosis virus (AMV) reverse transcriptase. A 20-μl reverse transcription reaction mixture containing 1 μg of total RNA, 1× reverse transcription buffer (10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1 mM deoxynucleoside triphosphates (dNTPs) 0.5 unit of RNase inhibitor, 0.5 μg of $oligo(dT)_{15}$, and 15 units of AMV reverse transcriptase were incubated at 42°C for 15 min, heated at 99°C for 5 min, and then incubated at 0-5°C for 5 min. PCRs were performed for 35 cycles with 3' and 5' primers based on the sequences of proto-oncogene primers (*c-jun*: 5'-AACGACCTTCTACGACGATG-3' and 5'-GCAGCGTATTCTGGCTATGC-3' *c-myc*: 5'-GTCACGACGATGCCCCTCAACGTG-3' and 5'-AAGTCCAAGTTCTGTCAGAAGGAA-3' *c-fos*: 5'-ACCATGATGTTCTCGGGTTTCAAC-3' and 5'-CTCTGTAATGCACCAGCTCAGTCA-3'). The GAPDH primers (5'-CTCCC AACGTGTCTGTTGTG-3' and 5'-TGAGCTTGACAAAGTGGTCG-3') were used as the internal standard. The signal intensity of the amplification product was normalized to its respective GADPH signal intensity.

2.9 Confocal Microscopy and Fluorescence Measurements

The measurement of cytosolic free Ca^{2+} concentration was estimated by the confocal microscopy analysis. Cardiomyocytes are plated on 4-well slide coated with 1.5% gelatin for 1 day in α-MEM containing 10% fetal bovine serum (Invitrogen) and 0.1 μM BrdU (Sigma Chemical). After incubation, the cells were washed with modified Tyrode's solution with 0.265 g/l $CaCl_2$, 0.214 g/l $MgCl_2$, 0.2 g/l KCl, 8.0 g/l NaCl, 1 g/l glucose, 0.05 g/l NaH_2PO_4 and 1.0 g/l $NaHCO_3$.Werethen loaded with 5 mM of the acetoxymethyl ester of fluo-4 (Fluo-4 AM, Invitrogen) for 20 min, in the dark and at 37°C. Fluorescence images were collected using a confocal microscope (Leica, Germany) excited by 488 nm line of argon laser and emitted light collected through a 510-560 nm band-pass filter. Relative data of intracellular Ca^{2+} was determined by measuring fluorescent intensity.

2.10 Statistical Analysis

Data were summarized from 3 individual experiments and expressed as means±S.E. Statistical analyses were performed by one-way ANOVA, using Bonferroni test for comparison of several groups. A value of $P<0.05$ was considered significant.

3. Results

3.1 ROS Production in H/R Cardiomyocytes

To show the ROS production in H/R cardiomyocytes as compared with I/R myocardium, ROS production was monitored at different times of reoxygenation in cardiomyocytes. And exogenously added H_2O_2 was chosen as positive control to show an increase in intracellular ROS. H (1 h) / R (3 h) caused a marginal increase in DCF fluorescence over the labeled normoxic control and hypoxic control cells. These data indicate that H (1 h) / R (3 h) condition expresses to meaningful ROS production (Fig 1A). In positive control, ROS production was significantly increased in H_2O_2 (200 μM) treatment for 30 min (Fig 1B).

3.2 Expression of G Proteins in H/R Cardiomyocytes and I/R Myocardium

It has been known that alteration of G proteins is related with adrenergic receptor. We were preliminarily examined that expression level of α_1-adrenoceptor was the highest in both H/

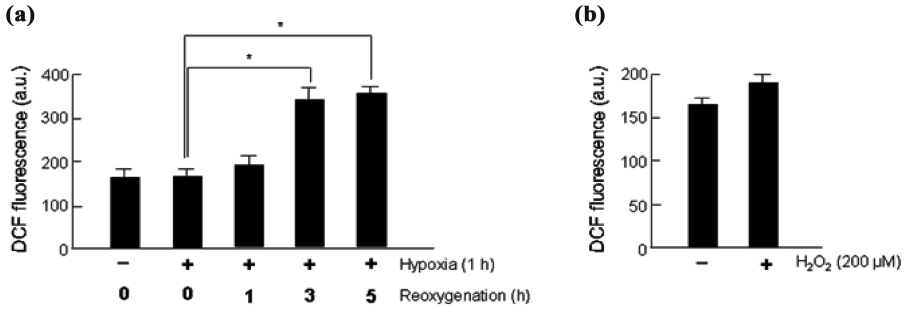

Figure 1. Reactive oxygen species (ROS) production in hypoxia/reoxygenated cardiomyocytes. (A) Neonatal cardiomyoctes subjected to hypoxia at reoxygenation time-dependent manner. ROS production was detected by fluorescence of H_2DCFDA (10 μM). (B) H_2O_2 (200 μM) was added in cardiomyocytes (*$p < 0.01$).

R cardiomyocytes and I/R myocardium among many subtypes of adrenoceptor (data not shown). To find whether the changes of G protein expression levels were involved with hypertrophy in H/R and I/R, expression level of the G proteins was examined. In H/R cardiomyocytes, G_h protein expression was mainly increased but the other G proteins expression were not hardly changed, compared to normal cardiomyocytes (Fig 2A). To address these phenomena *in vivo*, expression levels of G proteins were also measured in I (1 h) / R (3 h) myocardium. Like H/R cardiomyocytes, expression of G_h was significantly increased but the other G proteins was not changed in I/R myocardium (Fig 2B). Furthermore, G_h protein translocated in cellular membrane was more increased than cytosolic G protein after H (1 h) / R (3 h) (Fig 2C). Also increment of G_h protein by reoxygenation was time-dependently confirmed (Fig 2D).

3.3 Selectivity of G Protein in NE-Stimulated H/R Cardiomyocytes

Our previous study showed that G_h is partially involved in α_1-adrenergic receptor-stimulated cardiac hypertrophy independent of G_q regulation.[19] To further address the involvement of G_h in hypertrophic response of NE-treated

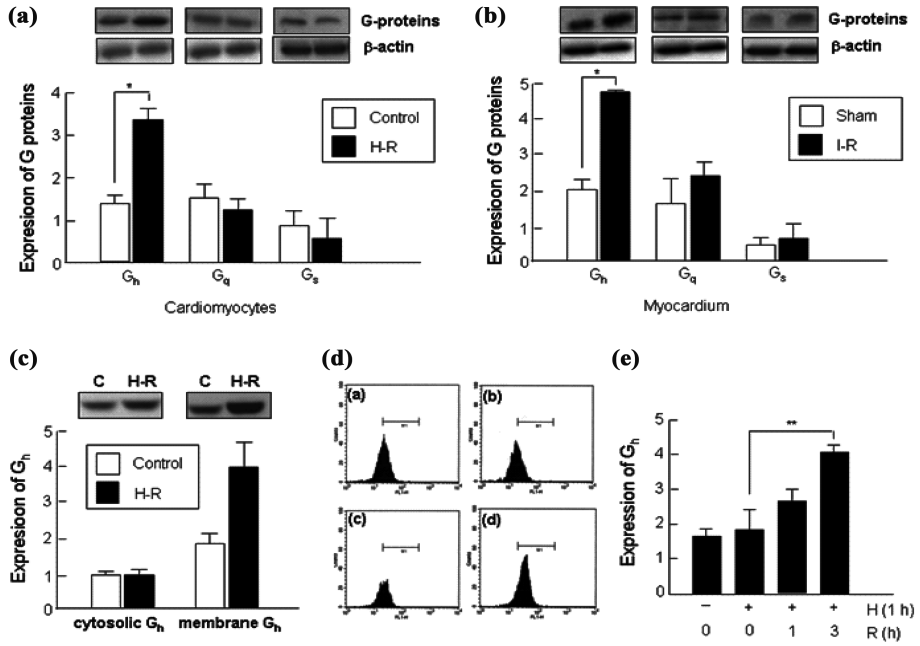

Figure 2. Expression of G proteins in H/R cardiomyocytes and I/R myocardium. Cardiomyocytes obtained from H (1 h) / R (3 h) (A) and heart tissues obtained from I (1 h) / R (3 h) (B) were performed Western blot analysis for expression of G proteins. (C) Expression levels of membrane and cytosolic G_h were also estimated in immunoblot analysis. (D) Expression level of G_h in H (1 h) /R (0 h, 1 h, and 3 h) cardiomyocytes was obtained in flow cytometry. (a) normal control, (b) H 1 h, (c) H 1 h/R 1 h, (d) H 1 h/R 3 h, and (e) Expression level of G_h (*$p < 0.01$ and **$p < 0.001$).

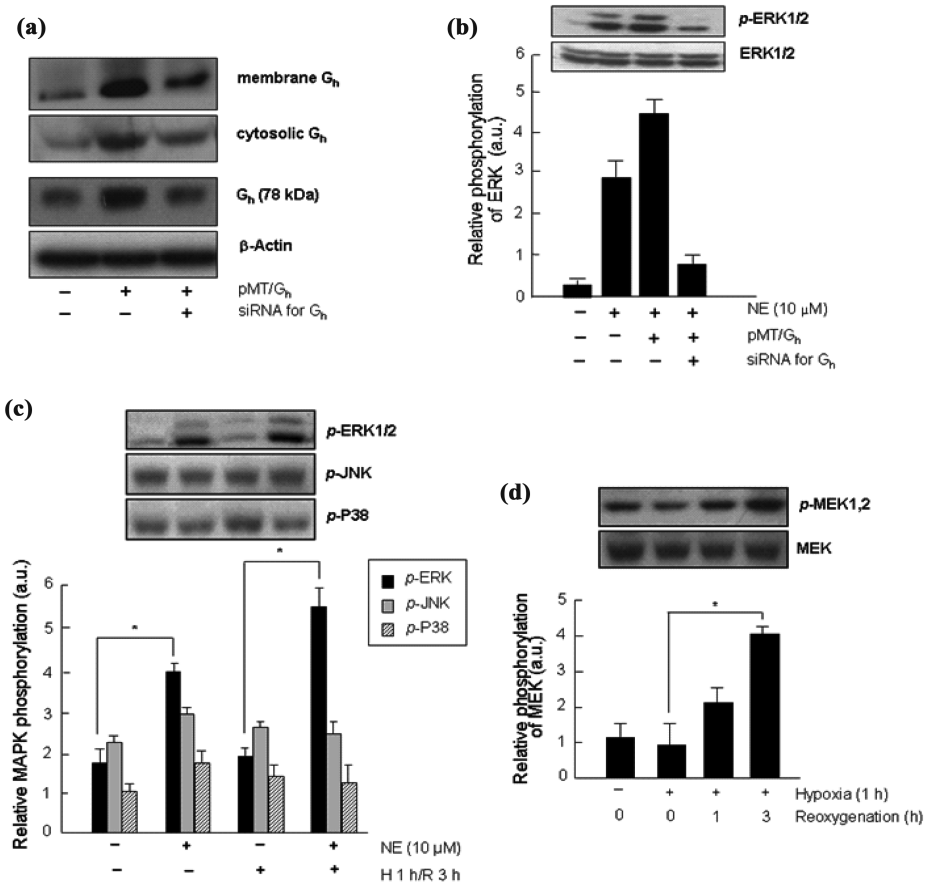

Figure 3. Changes between G_h and MAPK signaling pathway in the H/R cardiomyocytes. Cardiomyocytes were treated siRNA for 12 h and/or transfected pMT/G_h for additional 12 h and then NE stimulation was added for 10 min. G_h expression in membrane, cytosol, and total protein (A) and ERK phosphorylation (B) were detected by immunoblot analysis. (C) Activation of ERK, JNK and P38 was assessed under H (1 h) /R (3 h) with or without NE (10 μM) stimulation. (D) Cardiomyocytes were exposed to 1 h hypoxia followed by 1 h and 3 h reoxygenation. Phosphorylation of MEK1,2 was also detected by Western blot analysis (*$p < 0.01$).

cardiomyocytes, G_h gene was transfected into cardiomyocytes for overexpression. In G_h-transfected cells, G_h protein was highly expressed both in membrane and in cytosolic fraction (Fig 3A). NE further was increased phosphorylation of ERKs about 1.7-fold compared to the only NE-treated cell. To test if the increased phosphorylation of ERKs tightly relates to G_h overexpression in NE-stimulated cardiomyocytes, siRNA for G_h was transfected into cells for transient knock down of G_h. In cells treated with siRNA for G_h, NE-stimulated cardiomyocytes abolished phosphorylation of ERKs induced by α_1-AR (Fig 3B). These data indicate that H/R stimulation and I/R injury affect the expression of G_h, leading to ERKs phosphorylation.

3.4 Effect of MAPK Subfamily in NE-Stimulated H/R Cardiomyocytes

To test if overexpression of G_h induced by H/R affects MAPK pathway, expression and phosphorylation level of MAPK subfamily was examined in H (1 h) / R (3 h). As shown in previous data, increased G_h protein inducing H/R affected more highly on the hypertrophic marker MAPKs by NE stimulation in cardiomyocytes. The phosphorylation of ERKs, not p38 and JNK, was up-regulated by the treatment with H/R and NE (10 min) treatment, compared with NE-treated cells (Fig 3C). The phosphorylation of MEK1,2, upstream regulator of ERKs, was significantly increased in H (1 h) / R (3 h) (Fig 3D). These results show that intracellular signaling pathway induced by NE primarily processes by MEK1,2/ERKs cascade through α_1-AR in cardiomyocytes, and MEK1,2/ERKs cascade mediates to G_h protein increment through H/R.

3.5 Regulation of Proto-Oncogene Expression in H/R Cardiomyocytes

To determine the immediate-early response oncogenes in hypertrophy via H/R, we examined the mRNAs for *c-jun, c-fos,*

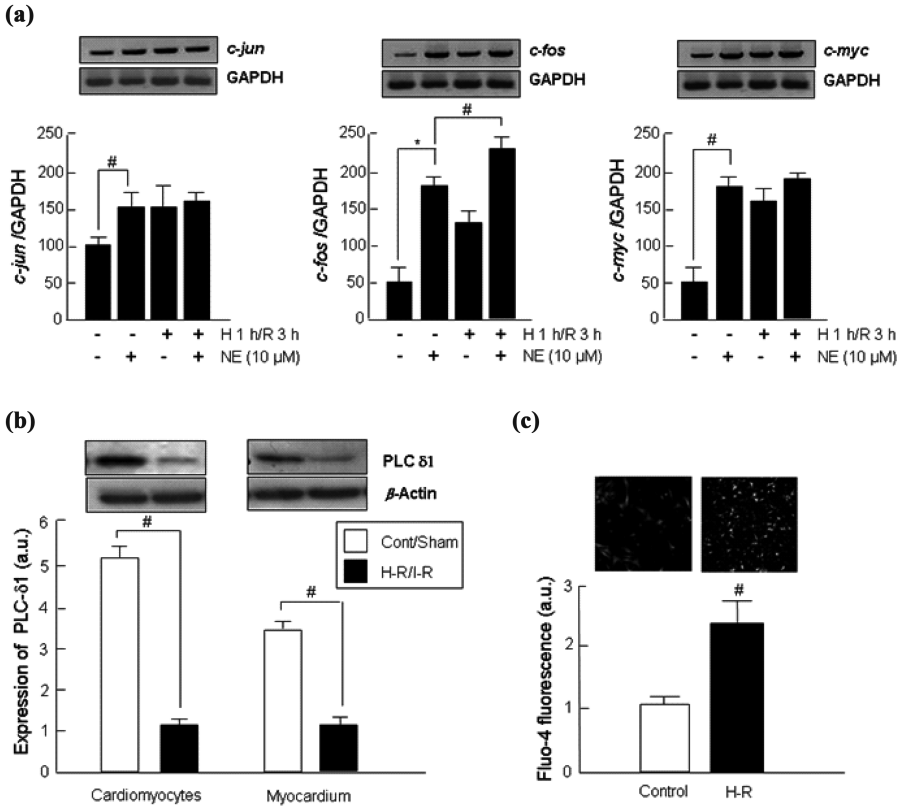

Figure 4. Specific regulation in proto-oncogene and PLC-δ1 expression in H/R cardiomyocytes. (A) The mRNA expression in cardiomyocytes was evaluated by RT-PCR analysis. Cardiomyocytes were 10 min stimulation with NE after H (1 h) / R (3 h). (B) Cardiomyocytes and heart tissues were each obtained from H (1 h) / R (3 h) and I (1 h) / R (3 h). Phosphorylation of PLC-δ1 was detected immunoblot analysis. (C) Cardiomyocytes were exposed to H (1 h)/ R (3 h) and representative $[Ca^{2+}]_i$ images were presented with fluo-4 fluorescence ($^{\#}p < 0.05$ and $^{*}p < 0.01$).

and *c-myc* in H/R cardiomyocytes. Significant increases in *c-jun*, *c-fos* and *c-myc* were observed by NE stimulation or H/R, but only *c-fos* was significantly elevated by H/R with additional NE treatment (Fig 4A). The mRNA levels of *c-jun* and *c-myc* were the independent consequence, the level of *c-fos* mRNA was increased after H/R and NE. It seems that *c-jun and c-myc* do not have involvement in hypertrophic response by oxidative modification of G protein. These data show that increased expression level of G_h in H/R mainly affects *c-fos* and activation of *c-fos* maybe provoke myocardial hypertrophy.

3.3 Expression of G Proteins on PLC-δ_1 in H/R Cardio myocytes and I/R Myocardium

We previously examined that PLC-δ_1 is the effector of G_h-mediated signaling. As the enzymatic activity of all PLC isozymes is dependent on Ca^{2+} concentration, the increment of intracellular Ca^{2+} may contribute to the increase in PLC-δ_1 expression.[20] But PLC-δ_1 was decreased in H (1 h)/R (3 h) and I (1 h)/ R(3 h) and did not coincide with elevated G_h protein (Fig 4B). Enhanced expression level of G_h by H/R or I/R was influenced by ROS production and not by increased intracellular Ca^{2+} concentration. These results mean that this hypertrophic mechanism involved in H/R and I/R was independent from pathway related to PLC δ_1.

4. Discussion

Cardiac hypertrophy is an essential process due to hemodynamic overload. Growth of hypertrophy is caused by increased workload, and NE, the primary transmitter of the sympathetic nervous system inducer, induces hypertrophy in numerous tissues including heart via interaction with GPCR to adrenoceptor and phospholipase C (PLC) and G_q protein mediates hypertrophic response in heart.[4,21] But, we and some groups have recently proposed that cardiac hypertrophy also related to the G_h pathway by α_1-AR stimulation.[19,22]

In this study, we identified that α_1-AR-mediated hypertrophy is more selectively mediated via the oxidative modification of

G_h during H/R or I/R injury. First of all, we primarily confirmed that mRNA levels of α_1-adrenoceptor subtype in H/R condition were not different and expressions of α_{1A}, a_{1B} and a_{1D} were not changed in I/R myocardium *in vivo* level (data not shown). These data indicate that hypertrophic response by ROS production was not related to the expression of α_1-AR each subtypes in both H/R cardiomyocytes and I/R myocardium.

Because of acute myocardial infarction, heart undergoes ischemic heart disease, and that is a major cause of mortality and morbidity. While reperfusion of ischemic myocardium is pivotal situation in myocardial salvage, reperfusion may also result in myocardial damage, according to cell swelling and disruption of mitochondrial membrane.[23] These mechanisms have included below. 1) cellular calcium loading, 2) no reflow phenomenon, and 3) oxygen radicals. Most importantly, ROS, unformation of oxygen radicals, is generated in ischemia and subsequent reperfusion. Isolated cardiomyocytes have experimented that hypoxia increases ROS production and damage of electron transport complexes.[24] While low levels of oxygen radicals importantly play in cellular homeostasis, differentiation and signaling.[25] Some studies demonstrated that receptor-stimulated coupling of the α_{1A}-adrenergic receptor to $G\alpha_{q/11}$ protein was increased in the tail arteries subjected to I/R.[26] Therefore, we also demonstrated in neonatal cardiomyocytes that α-AR-stimulated hypertrophy is associated with ROS-mediated activiation of G_h. The expression level of G_h was increased through ROS production, and increased G_h affects the development of myocardial hypertrophy in H/R (Fig 2 and 3).

And, we examined the signaling pathway on hypertrophy by G_h modification. After the exposure of the neonatal cardiomyocytes to I/R, NE stimulation leads to enhanced α_1-adrenergic-receptor-stimulated hypertrophy through enhanced G_h-MEK1,2-ERKs signaling and expression of *c-fos* (Fig 4A). During growth of cardiac hypertrophy, heart has been observed specific changes, 1) rapid induction of proto-oncogenes and heat shock protein genes (immediate-early genes), 2) quantitative and qualitative changes in gene expression, and 3) increased rate of protein synthesis.[2] The *c-fos* mediates both proliferative and cellular growth in many cell types. Recently, some researcher argued that the expression of *c-fos* was increased after I/R in brain tissue, also increased by hepatic I/R in rats, and stimulated by activation of PKC and this was related to increase of skeletal α-actin genes, β-MHC and MLC-2a which increase in hypertrophic response.[27-29] These results indicated that myocardial hypertrophy may involve in the early phase of myocardial H/R.

The hypertrophic signaling pathway via PLC was examined by many researchers. Some reports demonstrated that increased PLC-β_1 and -γ_1 activities during cardiac hypertrophic response and decreased PLC-γ_1 and -δ_1 activities during heart failure suggested an important action of PLC isozymes by volume overload of heart.[30] Hypertrophic response may be related to α_1/Gqa/PLC-β_1 signaling pathway.[31] The data in the study provide that hypertrophic mechanism related with $G_h$was independent from pathway related to PLC-δ_1 (Fig 4B and 4C). Thus, we revealed that PLC-δ_1 has no relation in hypertrophy via ROS modification of G_h.

Taken together, these findings indicate that α_1-AR stimulation by ROS cause the oxidative posttranslational modification of G_h in neonatal cardiomyocytes. Increased G_h enhanced hypertrophic response through the MEK1,2-ERKs signaling and *c-fos* expression. Thus, modification of G_h by ROS may play a role in the pathophysiology and/or therapy of myocardial remodeling.

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